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Canonical and non-canonical Hedgehog signalling and the control of metabolism

Raffaele Teperino^a, Fritz Aberger^b, Harald Esterbauer^c, Natalia Riobo^d, and John Andrew Pospisilik^{a,*}

^aDepartment of Epigenetics, Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany

^bDepartment of Molecular Biology, University of Salzburg, Salzburg, Austria

^cDepartment of Laboratory Medicine, Medical University of Vienna, Vienna, Austria

^dDepartment of Biochemistry and Molecular Biology and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

Abstract

Obesity and diabetes represent key healthcare challenges of our day, affecting upwards of one billion people worldwide. These individuals are at higher risk for cancer, stroke, blindness, heart and cardiovascular disease, and to date, have no effective long-term treatment options available. Recent and accumulating evidence has implicated the developmental morphogen Hedgehog and its downstream signalling in metabolic control. Generally thought to be quiescent in adults, Hedgehog is associated with several human cancers, and as such, has already emerged as a therapeutic target in oncology. Here, we attempt to give a comprehensive overview of the key signalling events associated with both canonical and non-canonical Hedgehog signalling, and highlight the increasingly complex regulatory modalities that appear to link Hedgehog and control metabolism. We highlight these key findings and discuss their impact for therapeutic development, cancer and metabolic disease.

Keywords

Non-canonical Hedgehog signaling; Metabolism; Development; Cancer

1. The Hedgehog signalling pathway

Proper control of development, tissue homeostasis and metabolism of higher multicellular organisms is a delicate and highly complex process that relies on the precise temporal, spatial and context-dependent regulation of molecular signalling pathways. The importance of controlled signal activation and termination is critical to the function of essentially all organisms. In higher organisms, the failure to induce, attenuate or stop signalling precisely

can result in severe developmental anomalies, metabolic disorders or life threatening malignant diseases [1–6].

Despite decades of molecular genetic advances, our understanding of the cues governing embryonic development and health of adult higher organisms comprises only a handful of signalling pathways. These include, but are not limited to Hedgehog, Wnt, Notch, Hippo, Jak/Stat, receptor-tyrosine kinases, and Tgf β signalling cascades [7]. While few, these signalling appear able to control a plethora of context dependent biological processes.

The Hedgehog signal transduction pathway can be regarded as a paradigm of how a single, and at first glance, simple molecular cue (i.e. secreted Hedgehog protein bound to its receptor) selectively translates into an array of distinct cellular reactions depending on context [8,9]. To date, a number of fundamental principles have been uncovered that link pathway activation and functionally distinct cellular response. For instance, Hedgehog can behave as a morphogen, that is, increasing concentrations of Hedgehog ligand can elicit distinct cellular responses [10,11]. Also, numerous studies have highlighted the importance of molecular cross-talk between the Hedgehog and other signalling cascades, interactions that can modulate signal strength and determine specificity of molecular and cellular phenotypes [12–23].

Somewhat unique to Hedgehog signalling are the number and variety of modes by which the Hedgehog signal can be stimulated and transduced to impact the behaviour and fate of the target cells [24]. For simplicity, the different modes of Hedgehog signalling are mostly commonly classified as either canonical or non-canonical. Canonical Hedgehog signalling in vertebrates is most similar to the Hedgehog signal transduction mechanisms originally identified in the fruit fly. They involve Hedgehog-dependent activation of the Gli zinc finger transcription factors [25,26]. Non-canonical Hedgehog signalling is less well defined; a multiplicity of different modes have been documented without any simple, over-arching theme. Simply put, non-canonical Hedgehog signalling currently refers to Hedgehog co-receptor dependent signals that do not clearly act via the canonical Hedgehog-to-Gli route (Fig. 1 and [24]). Non-canonical Hedgehog signalling pathways have been classified in two groups: *Type I* signals that stem from the peptide ligand receptor Ptch, and *Type II* signals that stem from the seven trans-membrane domain containing G-protein coupled receptor (GPCR) Smo. In addition, Smo-independent activation of Gli has also been referred to as non-canonical Hedgehog signalling.

Here, after a brief overview of canonical signalling, we will focus on Smo-dependent, Gli-independent non-canonical Hedgehog signalling. We will summarize recent findings on the role of Smo as a GPCR regulating cytoskeletal architecture, cell motility, and axon guidance, as well as highlighting a novel regulatory link to the maintenance of cellular and organismal energy homeostasis.

1.1. Canonical Hedgehog signalling

Canonical Hedgehog signalling was first discovered in *Drosophila*, where seminal work on embryonic patterning mutants led to the identification of the *hedgehog* gene. Loss of Hedgehog function in the fly results in a disorganized lawn of spiky processes and denticles

on the surface of the fly larva, a Hedgehog-like phenotype that coined the name of the pathway [27]. While canonical Hedgehog signal transduction is highly conserved, several key differences have emerged since the divergence of flies and mammals. Included, are a critical negative regulatory function of vertebrate Sufu, and an expansion of the activator and repressor repertoire of the fly transcription factor *Cubitus interruptus* to three distinct zinc finger transcription factors, Gli1, Gli2 and Gli3, in vertebrates [8,28–30]. The primary cilium, commonly thought to be a prerogative of Hedgehog signalling in vertebrates, has also been shown to play a central role in flies [31,32].

Vertebrate canonical Hedgehog signalling is initiated by binding of proteolytically processed and lipid modified Hedgehog ligand to its receptor Patched (Ptch), a twelve-pass transmembrane protein that represses the pathway in the absence of ligand [33–37]. Three distinct co-receptors, Cdo, Boc, and Gas1, facilitate high-affinity binding of mature Hedgehog ligand to Ptch, thereby enhancing Hedgehog signal strength [38–42].

Ligand binding to Ptch abrogates its repressive effect on the seven-pass transmembrane protein Smo, a key effector essential for canonical Hedgehog signal transduction [43]. The repressive role of ligand-free Ptch depends on its localization in the primary cilium, a single antenna-like structure that protrudes from the cell surface of most adherent cell types and functions as an organizer-like signal transduction compartment. Ciliary Ptch prevents pathway activation by blocking the entry of Smo into the primary cilium. Binding of Hedgehog protein to Ptch removes Ptch from the primary cilium, thereby allowing Smo to enter and, upon an unknown activation step, propagate the Hedgehog signal further downstream [28,44,45]. Despite intense efforts to understand Ptch function, the detailed mechanisms of how Ptch represses Smo in the absence of ligand is still elusive. Ptch contains a sterol-sensing domain and belongs to the family of RND (Resistance-Nodulation-cell Division) transporters [46]. Several functional studies support a model where Ptch prevents Smo activation either by removing Smo agonists such as oxysterols from the primary cilium or by increasing the influx of Smo antagonists into the cilium [47–50]. In addition, Ptch may also modify the lipid composition of Smo-containing endosomes and therefore negatively control Smo trafficking towards the primary cilium [51,52].

The key role of Smo in canonical Hedgehog signalling is to control the activation of the Gli zinc finger transcription factors [53]. Of note, the Gli family member Gli3, and to some extent also Gli2, exerts a dual function as transcriptional repressor (GliR) and activator (GliA) of Hedgehog target genes, where the two distinct functional states are controlled by proteolytic processing (reviewed in [2]). In the off-state of the Hedgehog pathway, Gli3 protein appears to continuously cycle through the primary cilium, where it is proteolytically cleaved into a C-terminally truncated repressor form lacking the transactivation domain. Gli3 repressor protein translocates to the nucleus, where it binds to the promoters of Hedgehog target genes to shut off transcription. The balance between Gli3 repressor and activator is tightly regulated by sequential phosphorylation and dephosphorylation events [54]. Kinases involved are PKA; Gsk-3 β ; and Ck1. The detailed mechanisms of how Smo activation in the primary cilium results in Gli activator formation are still unclear [55–61]. Recent studies suggest that binding of Hedgehog to Ptch removes the ciliary G-protein

Coupled Receptor Gpr161 [62,63]. Gpr161 in the cilium inhibits Hedgehog signalling via PKA and Gli3 repressor formation [62].

Sufu is a critical negative pathway regulator in vertebrate Hedgehog signalling and prevents nuclear translocation of Gli proteins [29,64]. As a consequence of Sufu degradation and Gli-Sufu dissociation, Gli activator forms translocate to the nucleus and activate the expression of target genes, which includes a self-amplifying loop via induction of Gli1 itself [65–69].

1.2. Smo-dependent non-canonical Hedgehog signalling

Type II non-canonical Hedgehog signals are defined as Smo-dependent, Gli-independent cascades that respond to Smo agonists and elicit cellular responses, ranging from Ca^{2+} signalling and cytoskeletal rearrangement to metabolic rewiring (see Fig. 1).

The Hedgehog co-receptor Smo has 7-transmembrane (TM) domains. This topology resembles that of G protein-coupled receptors (GPCRs). The GPCR superfamily is subdivided into five families sharing homologous domains in the extracellular N-terminal region and intracellular C-terminal tail and having similar types of ligands, such as small molecules, peptides and glycosylated proteins [70]. Smo belongs to the F group, which also contains the Frizzled (Fz) isoforms, and has a characteristic extracellular cysteine-rich domain (CRD) and a long intracellular tail. Interestingly, the crystal structure of the 7TM bundle, despite a mere 10% sequence homology, highly resembles that of group A GPCRs, which use peptides as ligands [71] and also have an eighth short intracellular α -helix that lies parallel to the lipid bilayer. Smo is also characterized by large extracellular loops that make contacts with the CRD linker region and with TM helix III through a network of disulfide bonds [50]. The CRD is a compact globular domain, highly homologous to that of Fz receptors, containing four α -helices and 2 short β -barrel motifs. The CRD and the extracellular loops make a lid that partly occludes the binding pocket of one type of Smo small molecule modulators: the agonist SAG and the selective partial agonist/antagonist cyclopamine and its derivatives [50,72]. Oxysterols, small agonists of Smo, have been mapped to bind to the CRD, which allosterically modulates the cyclopamine-binding pocket [73,74]. Since some natural oxysterols bind to and activate Smo, they have been proposed as Smo endogenous ligands [47–49]. However, a recent study shows that oxysterol binding mutants, abrogating Smo activation by exogenous oxysterols, fail to suppress Shh or SAG agonism in vivo [50]. Thus, so far Smo is an orphan GPCR, making the “R” of GPCR potentially misleading.

Regardless of the orphan nature of Smo, there is clear evidence that it activates heterotrimeric G proteins, like all GPCRs. Biochemically, Smo catalyzes the GDP-GTP exchange of all members of the G inhibitory (G_i) family of G proteins (G_{i1} , G_{i2} , G_{i3} , G_o , and G_z) but not of any other G protein families when expressed in the absence of Ptch or when activated by the small agonist purmorphamine [19]. Smo exists as a homodimer making contacts through both the N-terminus and the C-terminus domains, as reported for many GPCRs [75]. The long C-tail of Smo undergoes a conformational change upon activation when the strong positive charge of an Arg-rich stretch is neutralized by phosphorylation of a large number of Ser/Thr residues [75]. Interestingly, the C-tail partly precludes interaction with G_i proteins, since a C-tail deletion significantly increases $\text{GTP}\gamma[\text{S}^{35}]$ binding, thus

suggesting that active Smo is in a less compacted quaternary structure that allows G protein interaction [19]. Supporting Smo-G_i coupling, the strong selectivity and potency of SMO towards the G_i family is similar to the prototypical serotonin receptor 5HT1aR G_i-GPCR. That said, Smo exhibits greater inhibitory potency for cAMP production [76]. Noteworthy, Smo-dependent G_i activation is not sufficient to trigger canonical Hedgehog signalling downstream of Smo, and in some cell types is not even necessary [19,77]. The long intracellular C-tail of Smo is required for phosphorylation by Grk2 and Ck1, for β -arrestin binding, trafficking to the primary cilium, and for interaction with Costal2 and Fused in *Drosophila* [78–84]. Therefore, it is possible that the conformational change of Smo allows interaction with G_i proteins regardless of plasma membrane vs. primary cilia membrane localization.

The capacity of Smo to act as a classic GPCR towards G_i proteins is evidenced in several contexts. Activation of Gli-dependent transcription is maximal in the presence SMO-G_i protein coupling in some cell types but not in all [77]. Specifically, the use of a Pertussis toxin (PTX), a small ADP-ribosylase that modifies an N-terminal Cysteine of G α_i thus preventing GPCR interaction, has served to demonstrate that Gli activation in NIH3T3 cells, CH10T1/2 cells, and cardiomyocytes is largely sensitive to inhibition of G_i protein activation ([19,76], N.A.R. *unpublished data*). Conversely, in mouse embryonic fibroblasts (MEFs) Smo-G_i coupling is not essential for canonical Hedgehog signalling, and the reason is unclear [77]. In *Drosophila* S2 cells, depletion of the single G_i family member by siRNA prevents activation of Ci and lack of G_i in the wing disc inhibits expression of the Hedgehog-target gene *dpp* [85]. PTX has also been used with conflicting results in in vivo studies in zebrafish and avian embryos. In zebrafish, PTX phenocopies many aspects of Shh deficiency including cyclopia, lack of forebrain ventral specification, and an expansion of the sclerotome at the expense of adaxial fates in the posterior somites [86]. In the chick embryo neural tube electroporation model, PTX did not affect gross dorso-ventral patterning and thus it was concluded that G_i is not required for Hedgehog-dependent patterning [87]. However, the nervous system expresses G_z, a member of the G_i family that couples to Smo but is insensitive to PTX because it lacks the Cys residue that is the substrate for ADP-ribosylation. Therefore, the results remain inconclusive with respect to the role of G_i proteins in neural tube patterning by Shh. Proliferation of mammalian cerebellar granule neuron precursors in response to Shh, mediated by Gli transcription, also depends on G_i proteins as it is inhibited by PTX and by siRNA-mediated depletion of G α_{i1} , G α_{i2} , or G α_{i3} . Interestingly, in the latter model, overexpressed G α_{i1} and G α_{i2} were detected in different regions of the primary cilium [88].

In the past five years, numerous pieces of evidence emerged supporting a key role of Smo/G_i coupling in non-canonical Hedgehog signalling. Recent studies reported that rapid activation of Rac1 and RhoA by Shh or a Smo agonist leads to migration in fibroblasts and cholangiocarcinoma cells and to tubulogenesis in endothelial cells [89–91]. This response is dependent upon Smo coupling to G_i, as it is sensitive to PTX and does not require the C-terminal domain of Smo nor Smo translocation to the primary cilium [90,92]. The use of PTX has also been essential to conclude that in spinal neuron precursors activation of Smo leads to increased calcium spike activity through a nifedipine-sensitive calcium channel

[93]. Furthermore, the role of non-canonical Hh signalling utilizing Smo as a GPCR in the regulation of metabolism, as well as the pharmacology governing its function, have been recently reported [72].

Activation of G_i proteins by Smo has also been linked to activation of the NF- κ B transcription factor in diffuse B-cell lymphoma cells [94]. In those cells, activation of Smo enhances its interaction with G_i proteins and promotes the recruitment of a signalling complex containing CARMA, Bcl10 and MALT1, which in turn initiate activation of p65/p50 NF- κ B. The mechanism is rapid and independent of Gli-dependent transcription. Remarkably, this study and a previous one also provided evidence for G_{12} activation by Smo [95]. Our studies using the clean background of Sf9 cells and in HEK293 cells however indicate that Smo does not have the capacity to activate either G_{12} or G_{13} [19,76]. Maximizing stoichiometry and proximity of Smo and G_{13} via fusion of the C-tail to the α subunit of G_{13} , a strategy commonly used when studying GPCRs, cannot induce G_{13} activation, while the same fusion to $G\alpha_i$ strongly promotes G_i activation [76]. A possibility is that Smo is activating G_{12} in those contexts via heterodimerization with another GPCR in a cell type specific manner, or that the interpretation was confounded by the fact that other GPCRs that stimulate G_{13} are able to increase Gli-transcriptional activity independently of SMO [96].

Smo is regulated by all tested inhibitors/agonists (cyclopamine, KAAD-cyclopamine, SANT-1, tomatidine, purmorphamine) at a similar concentration when Gli transcriptional activity and G_i protein activation are used as readouts [19,97–99]. This evidence, thus, suggests that the active conformation of Smo for G_i protein coupling and Gli activation is the same. In addition, the lack of primary cilia in the vesicularized membranes of Sf9 cells (where the GTP binding assays were performed), as well as the finding of cilium-independent G_i protein-dependent Smo signalling in fibroblasts, suggests that Smo- G_i interactions are not confined to the primary cilium. However, Smo- G_i interaction within the primary cilium could occur in a cell type dependent manner. For instance, the metabolic switch induced by Smo activation in adipocytes and skeletal muscle is dependent on both G_i protein and translocation to the primary cilium [72]. In addition, $G\alpha_{i2}$ and $G\alpha_{i3}$ have been shown to localize to primary cilia in the context of Shh-induced proliferation of cerebellar granule precursor neurons [88].

Altogether, the burden of evidence indicates that Smo couples to heterotrimeric G proteins of the G_i family. The significance of that interaction is more obvious in non-canonical Hedgehog signalling, but it seems to be permissive for Gli activation in some cell types, possibly due to the consequent drop in cAMP levels and PKA activation.

2. Hedgehog signalling and metabolism

The connections between Hedgehog signalling and metabolism are multiple, and they span processes ranging from ligand maturation and secretion, to receptor activity and cellular and organismal responses (see Fig. 2). Work by ourselves and others suggest the connection is bi-directional: that organismal metabolic state modulates Hedgehog signalling and that Hedgehog signalling regulates cellular and organismal metabolism.

2.1. Metabolism regulates Hedgehog

Active Hedgehog proteins (Shh, Dhh and Ihh) carry lipid moieties. They are fatty acid and cholesterol modified at the N- and C-termini respectively and these modifications are essential for their maturation, secretion and biological activity [39,100–107]. Lipid-modified Hedgehogs have high affinity for the cell membrane and localize to lipid-raft microdomains. The current model is that fatty acylation targets Hedgehog to the membrane of the secretory cell, while cholesterol anchors them to the rafts of both secretory and receiving cell, thus preventing inappropriate secretion and increasing stability at the target cell [108,109].

The Hedgehog pathway is also sensitive to intracellular and extracellular sterols. Specifically, hydroxylated cholesterol derivatives known as hydroxysterols (OHCs) act as Smo agonists. Structure–Activity–Relationship (SAR) studies have highlighted two necessary features for OHCs to be effective Smo agonists: (i) stereoselectivity – only the (S) stereoisomer is active [110,111]; and (ii) hydroxyl-side chain substitution – long aliphatic chains and a free additional hydroxyl moiety on C6 increase activity [73,110]. Several of these hydroxysterols, including 20(S)OHC, 25OHC, 7-keto-25OHC and 7-keto-27OHC allosterically modulate Smo activity by binding either inside or outside of the SAG-binding pocket [49,50,73]. Also, pharmacological depletion of endogenous cellular sterols impacts Smo-mediated signalling [112] and cyclopamine, the prototypical competitive Smo inhibitor, is an alkaloid with a sterol-like structure [97]. Currently, the idea is that intracellular sterols inhibit, while extracellular sterols activate, Smo activity.

Sterol-dependent Smo modulation has been attributed thus far to the sterol-sensing domain present on Ptch1, which is essential for the Ptch1-dependent tonic inhibition of Smo [46,113–115]. That said, the Smo-CRD has been recently identified as the site of action for several oxysterols [73] and appears essential for oxysterol-dependent Smo modulatory activity. Interestingly, interaction events at the SAG-binding pocket and the CRD are independent. While resistant to Hedgehog-mediated pathway activation, CRD mutants are fully responsive to SAG and cyclopamine [50]. These results suggest physiologically independent, though equally important, sterol-sensing functions for both Ptch1 and Smo. Further supporting a sterol-sensing function for Smo, Lrp2 has been identified as a Smo co-receptor [116]. Lrp2, also known as Megalin, is a widely expressed 500KDa transmembrane protein that binds disparate substrates [117] and participates in their endocytosis and eventual lysosomal degradation [118,119]. Lrp2 acts as a Shh-binding protein in the forebrain where it sequesters Shh to its site of action and controls Shh-Ptch1 internalization and degradation [116]. LRP2 knockout mice phenocopy Hedgehog signalling mutants exhibiting anomalies in forebrain development [116].

In addition to sterols, intracellular lipids can also activate Smo, as is the case for phosphatidyl-inositol-4-phosphate (PI4P) [120]. PI4P is a polar lipid mainly synthesized and localized to the Golgi membrane, where it contributes to vesicle budding and intracellular trafficking [121]. Recent findings also indicate roles for PI4P at the plasma membrane, where it contributes to the pool of polyanionic lipids that define plasma membrane identity [122]. In *Drosophila melanogaster*, PI4P accumulation facilitates Smo activation, most likely by promoting its trafficking to the plasma membrane [120], and indicative of a

feedback system, PI4P levels are controlled by Ptch1 signalling [120]. Interestingly, PI4P synthesis and degradation are dynamically regulated by kinases and phosphatases whose loss of function leads respectively to inhibition or activation of Hedgehog signalling [120]. In *D. melanogaster* and mammals, Hedgehog proteins, hydroxysterols, and Smo modulatory lipids all travel on lipoproteins [123–127]. One hypothesis is that the Ptch1/Smo/Lrp2 receptor system anchors circulating lipoproteins to the cell surface, placing active Hedgehog ligands in close proximity their binding site on Ptch1. This “Lipoprotein bridge”, as well as the sterol-mediated Smo allosteric modifications, may modulate pathway activity by either facilitating the Hedgehog-dependent release of Ptch1 or by inhibiting Smo activity through lipoprotein-loaded inhibitory ligands. Noteworthy, Hedgehog proteins travel on VLDL in humans [128]. Intriguingly, VLDL dimensions (diameter 30–80 nm) are compatible with protein dimerization and membrane receptor clustering. We refer the reader to the review by S. Eaton et al. in this same issue for a detailed review of these ideas.

2.2. Hedgehog regulates metabolism

The Hedgehog signalling pathway is essential for proper embryonic development [11] and thought to be mostly quiescent in adults. Physiological reactivation of the pathway occurs in stem cell compartments as a response to tissue damage and drives cell fate transitions, trans-differentiation and metabolic reprogramming [129]. Also, genetic and pharmacological modulation of the pathway impacts adipose tissue development [4,130–133] and function [72,134,135]. Importantly, inappropriate reactivation of the pathway is associated with disparate human cancers [3,9,14,21,69,136–143] spanning almost every tissue type, including liver, pancreas, brain, stomach and intestine. Most of these effects have been associated with canonical, Gli-dependent, Hedgehog signalling pathway. Non-Canonical, Gli-independent, pathway(s) instead have been shown to play a prominent role in Ca^{2+} signalling, cytoskeletal rearrangement, axon guidance and in adipose tissue and skeletal muscle metabolism [72,77,89,90,93,144].

Below, we try to summarize these findings and put them into perspective. It is clear that while highly interesting, much work remains before we have clear understanding of the mechanisms and consequences of Hedgehog signalling activation in adult physiology and disease.

2.2.1. Liver—The liver is a vital organ for vertebrates. It participates in many physiological processes including detoxification, energy metabolism and digestion. The liver is composed of numerous cell types, each with its own specialized function. In addition to the parenchymal hepatocytes, the non-parenchymal compartment includes endothelial cells, Kupffer cells, cholangiocytes, hepatic stellate cells (HSC) and myofibroblasts.

The liver is the major source of the Hedgehog-carrying VLDL particles in mammals [145]. VLDL contain triglycerides, phospholipids, cholesterol and cholesteryl esters and transports them throughout the body [146,147]. Clinically, elevated triglycerides are associated with insulin resistance, non-alcoholic fatty liver disease (NAFLD), excess alcohol consumption, liver cirrhosis, hepatocellular carcinoma (HCC) and much more. Interestingly, upon liver damage, the secretion of Hedgehog ligands is upregulated in cholangiocytes and

myofibroblasts and sustains proliferation and survival in both cell types [148,149]. In response to damage, HSCs undergo an Epithelial-to-Mesenchymal-Transition (EMT) and trans-differentiate into myofibroblasts (MF), resulting in liver fibrosis. Activation of the Hedgehog pathway in HSCs, for example downstream of Leptin signalling [150], is responsible for HSC to MF trans-differentiation [150]. This cell fate transition is intriguingly dependent upon Gli transcription factors, Hif-1 α and a metabolic switch towards aerobic glycolysis in HSCs [151]. These findings represent the first evidence of a direct implication of canonical Gli-dependent Hedgehog signalling in glycolytic reprogramming of cellular metabolism. Strikingly, this phenomenon seems to be a common feature of liver damage-induced Hedgehog signalling, as it has been shown in other forms of non-carcinogenic liver damage [149,152–156].

The HCC scenario is rather unique. Here, Hedgehog ligands are secreted by malignant hepatocytes, induce Gli-dependent Warburg reprogramming and lactate secretion in stromal cells (mainly myofibroblasts) and lactate signals back to malignant hepatocytes to sustain their growth and survival [137]. This metabolic reprogramming is known as the “*Reverse Warburg*”. First introduced in 2009 [157], a *reverse Warburg effect* describes the ability of cancer cells to induce a Warburg effect in neighbouring stromal cells and benefit from the secreted lactate to sustain their proliferation and survival [157–161]. Thus, damage-induced Hedgehog signalling in the liver leads to metabolic reprogramming and cell fate transition in HSCs and MFs, thus leading to conservative and/or pro-oncogenic consequences.

As a different form of liver damage, Hepatitis-C Virus (HCV) infection activates the Hedgehog signalling pathway in human hepatocytes [162], where Hedgehog activation has been shown to facilitate HCV secretion and replication [163]. Interestingly, the HCV replication-secretion cycle involves the intracellular pool of PI4P and takes advantage of the VLDL secretory machinery [164–166]. Besides damage-induced response, Hedgehog signalling is also active in healthy hepatocytes and contributes to Igf-1 homeostasis [167].

The findings presented above indicate a prominent role for Hedgehog in liver physiology and pathology. From a metabolic point of view, the liver features a so-called “*Metabolic Zonation*”. Different metabolic pathways are carried out in different zones of the hepatic lobules, allowing liver to progressively or selectively cope with the diversity of the metabolic challenges [154]. Molecular mechanisms of liver metabolic zonation are not clear. The Wnt/ β -catenin signalling pathway has been proposed as the master regulator [168,169] and indirect evidence has led scientists hypothesize a prominent role for Hedgehog too [154].

2.2.2. Pancreas—The pancreas is a two-headed organ, which presents endocrine function (in relation to the control of blood glucose homeostasis) as well as gastrointestinal function as an exocrine organ. The endocrine function of the pancreas is achieved by four highly specialized cell types within the islet of Langerhans. These include: (i) alpha cells, which secrete glucagon, which stimulates hepatic glucose production and gluconeogenesis; (ii) beta cells, which secrete insulin, the master regulator of blood glucose homeostasis; (iii) delta cells, which secrete somatostatin, a hormonal brake on alpha and beta cell activity; and (iv)

F-cells, which secrete pancreatic polypeptide in response to food intake to modulate endocrine and exocrine pancreatic functions.

Hedgehog signalling controls development of both endocrine and exocrine pancreas, as well as their function in adults. Furthermore, inappropriate activation of the pathway, alone or in combination with other oncogenes is associated with the development of pancreatic adenocarcinoma (PDAC) [139,170–172].

During embryonic development, the endodermal foregut gives rise to the anterior part of the digestive canal from the mouth to the duodenum. The pancreas develops from the pancreatic bud of the endodermal foregut and its surrounding mesoderm. Staining of embryos at E10.5 shows that Shh is expressed throughout the gut endoderm, with the exception of the pancreatic bud [173]. Interestingly, when Shh is constitutively overexpressed in the pancreatic bud under the control of the *Ipfl/Pdx1* promoter the pancreatic mesoderm differentiates into smooth muscle and interstitial cajal cells typical of the developing intestine rather than into pancreatic mesenchyme and spleen [173]. These data indicate, therefore, that endodermal-derived Shh directs differentiation of the surrounding mesoderm and that tight spatial and temporal restriction of Hedgehog signalling is critical for proper development of the digestive tract.

The role of Hedgehog signalling in the pancreatic epithelium, though, is debated. Pancreatic epithelial cell-specific activation or inhibition of the pathway has only mild developmental effects, which suggest a role for Hedgehog signalling activation mainly in the pancreatic mesenchyme [170,173–177]. As an example, Pdx1-driven depletion of Smo in pancreatic epithelial progenitor cells results only in a transient delay in beta-cell development [177]. Interestingly, while beta-cell numbers recover after birth they are hypofunctional and Pdx1-Smo^{-/-} mice develop a mild insulin-dependent diabetes, characterized by glucose intolerance, increased insulin sensitivity and reduced insulin secretion [177]. This work confirmed a previous study [178], which showed that canonical Hedgehog signalling controls insulin transcription and secretion.

The mild responsiveness of pancreatic epithelial cells to constitutive activation of Hedgehog has been suggested to be linked to the primary cilium, a structural requirement for signalling downstream of Smo [179]. Adult epithelial cell-specific over-activation of the pathway, together with primary cilium ablation, leads to pancreas dedifferentiation, with striking re-expression of progenitor markers and reduction of the endocrine area. Double transgenics are metabolically compromised and develop age-dependent insulin-negative undifferentiated pancreatic cancers [176,179]. So, while well-implicated in pancreatic form and function, a clear role for Hedgehog signalling in normal pancreatic physiology begs deeper investigation.

2.2.3. Muscle and adipose tissue—Until the 1980s adipose tissue was primarily viewed as a simple site of calorie storage. With the discovery of adipokines (adipocytes released cytokines), this view radically changed, and the concept was born of adipose tissue as an endocrine organ at the centre of energy homeostasis. The use of lipid droplet as a means of energy storage is conserved in all eukaryotes from worms to humans. In mammals,

two grossly different adipocytes are recognized: (i) *white* adipocytes, which contain a single lipid droplet with hormone-sensing and lipid storing and releasing functions; and (ii) *brown* adipocytes, mitochondrial-dense, multilobular adipocytes, which burn lipids and produce heat via largely uncoupled respiration. White and brown adipocytes reside in different and differently distributed depots, namely white (WAT) and brown (BAT) adipose tissues and have largely different gene expression signatures and developmental origins [180].

The role of Hedgehog signalling in adipocyte differentiation and adipose tissue biology has long been debated. Since 1995, there have been indications sustaining a role for the pathway in stimulating and inhibiting adipogenesis [4,130,131,181–185]. The current notion, though, is that activation of the pathway in white adipocyte precursors has a general inhibitory role on adipogenesis from flies to mice [4,130].

Hedgehog acts upstream of PPAR γ to channel preadipocytes fate away from adipogenesis towards osteogenesis. This is achieved via Gli-dependent induction of anti-adipogenic transcription factors, with concomitant inhibition of the pro-adipogenic ones [4,130]. Interestingly, Hedgehog-dependent adipogenesis block is restricted to white adipocyte progenitors. aP2-Sufu knockout mice (which feature constitutive activation of the pathway in both white and brown fat depots) display a white adipose tissue-specific lipoatrophy, with a fully developed and functionally intact brown adipose tissue depot [4]. Thus, Hedgehog was one of the first hormonal axes identified capable of differentially regulating white and brown adipogenesis.

Worthy of mention, aP2-Sufu^{-/-} mice feature a unique healthy lipoatrophic phenotype with no signs of metabolic abnormalities. Even more surprisingly, the remaining white adipose tissue (roughly 10 per cent of a fully developed white fat mass) in these mice takes up 3-fold more glucose than that of wild-type animals and has morphological and genetic properties of the energy burning brown adipose tissue. Thus, constitutive, fat-specific activation of Hedgehog (i) selectively blocks white adipogenesis; (ii) activates metabolism in mature, fully developed, white adipocytes; and (iii) shifts white adipocytes towards brown-like functionality.

In an independent study using an oncogenic, constitutively active mutant of Smo, Smo^{M2}, under control of aP2-Cre mediated expression, Hatley et al. observed aggressive head and neck tumours transcriptionally and histologically reminiscent of embryonic rhabdomyosarcoma (ERMS) [134]. ERMS is the most common soft tissue malignancy in children, commonly associated with hyper-activation of canonical Hedgehog signalling. Though a tumour of muscle-like appearance, the cell-of-origin for ERMS has not yet been identified. While speculated to be of brown adipocyte progenitors lineage in the case of the aP2^{Cre} Smo^{M2} model, a caveat remains in that aP2-Cre has been shown on multiple occasions to be promiscuous in its expression. Activation of Hedgehog signalling is believed to be critical for muscle development in zebrafish [186–189]. In higher eukaryotes, Hedgehog promotes proliferation, commitment and differentiation of adult muscle cells [190–194] as well as muscle regeneration upon physical and ischaemic injury [195–198]. Surprisingly, Smo^{M2}-driven Hedgehog activation in muscle satellite cells fails to induce ERMS [134,196,199].

So, with respect to a unified role in adipose, questions still remain: activation achieved by Su(fu) deletion leads to white-specific lipoatrophy with an apparent white-to-brown adipocyte transition [4]; upstream over-activation by Smo^{M2} using the same Cre driver leaves adipose tissue development unimpaired, but induces fat-to-muscle lineage transition and cancer [134]. Could unidentified Smo-dependent or Smo^{M2}-specific secondary signalling pathways be involved? These questions remain open.

3. Non-canonical Hedgehog signalling regulates energy metabolism

The studies above indicate a clear role for canonical, Gli-dependent signalling in regulating metabolism and/or metabolically relevant tissues. Below we discuss the data that implicates non-canonical Hedgehog signalling as a novel rheostat linking acute nutrient status and energy homeostasis.

3.1. General features and molecular signature

As mentioned above, non-canonical Hedgehog pathways are defined as signalling originating from Ptch1 (*Type I*) and/or Smo (*Type II*) and independent from the Gli transcription factors [200].

We recently reported a novel *Type II* non-canonical pathway which rapidly rewires metabolism in vitro and in vivo [72]. Initially identified in differentiated 3T3L1 adipocytes, Smo activation by ligand binding, and/or Ptch1 inactivation, is coupled to a rapid L-type-dependent Ca²⁺ influx. Subsequent activation of Camkk2 then triggers phosphorylation and activation of Ampk, a sensor of cellular metabolic state and one of the master regulators of cellular metabolism (for a review see [201]). The result is a rapid, insulin-independent glucose uptake and a shift away from mitochondrial oxidative phosphorylation towards aerobic glycolysis -a metabolic signature reminiscent in many ways of the hallmark Warburg effect observed in many cancers [202,203]. Downstream signalling determinants of this glycolytic shift are the oncogenic slow-acting isoform of Pyruvate Kinase, Pkm2, and the rate-limiting enzyme of the Krebs cycle, Pdh1. Both Pkm2 and Pdh1 play important roles in aerobic glycolysis in cancer [204–209]. Tyrosine phosphorylation regulates their activities such that intracellular pyruvate accumulates and is then converted into lactate by the NAD⁺-producing Lactate Dehydrogenase.

For Hedgehog biologists, this novel non-canonical branch highlighted two noteworthy characteristics: (i) the notion of selective partial agonism; and (ii) a spatial restriction to the basal body of the primary cilium.

Inappropriate activation of the Hedgehog signalling pathway is associated with numerous disparate human cancers, ranging from haematological malignancies to brain and skin cancers and solid tumours of the lung and the intestinal tract [3,9,14,21,69,136–143]. At least seven pharmaceutical companies worldwide are developing Hedgehog antagonists and, many of them – those that target Smo – are in advanced clinical development. Among them, the Genentech-produced Vismodegib (or GDC-0449) was approved by the FDA in 2010 and is now the treatment of choice for locally advanced and metastatic basal cell carcinoma

[138,210,211] – the most common non-melanoma skin cancer associated with constitutive activation of the Hedgehog signalling pathway [143,212,213].

Among Smo binding small molecules, several modes of inhibition exist. Cyclopamine and Vismodegib (among others), for example, allow Smo translocation to the primary cilium, but prevent its movement to the tip of the cilium and thus block signal transduction. Molecules of the SANT family (SANT-1, 2 and 3) block Smo at an earlier step, preventing translocation and entry to the primary cilium. Intriguingly, when probed against the ability to inhibit the metabolic non-canonical Hedgehog signalling, only molecules of the first class behave as agonists, inducing Ca^{2+} influx, Ampk phosphorylation and glucose uptake with a potency 10-fold higher on average than that required for their own inhibitory effects on canonical signalling. Smo agonists, which induce translocation to the base and subsequently to the tip of the cilium also induce the non-canonical signal, albeit at somewhat reduced amplitude [72]. These findings indicate first, that canonical and non-canonical signalling cascades can be uncoupled at the level of Smo, and second, that the metabolic non-canonical signalling arm requires Smo translocation to the base of the primary cilium. This interpretation is underscored by the fact that Smo-dependent Ampk activation requires a fully intact primary cilium, and that CLD-Smo mutant fails to induce it.

The primary cilium is a microtubule-based organelle with crucial roles in vertebrate development [31,214] and human genetic diseases [215,216]. Impaired cilium function or development is associated with a group of disorders known as ciliopathies and characterized by developmental defects and interestingly, obesity. Specifically, the AMPK activation step downstream of Smo is compartmentalized to the basal body of the primary cilium, where Ampk associates directly with its activating kinase Lkb1 [217]. The findings that Smo-CLD or other variants unable to localize to the cilium (e.g. Smo^{M2}) are unable to activate the Smo-Ampk axis implies that the pathway, though compartmentalized to the basal body, still requires an intact primary cilium.

3.2. Therapeutic and physiological relevance

Hedgehog signalling is a pro-oncogenic pathway. Therefore, the use of Hedgehog agonists in therapy has always been disregarded, despite beneficial potential in a series of diseases [110]. The finding that canonical antagonists were partial agonists of the metabolic pathway, prompted us to further investigate the glucose uptake effects. Notably, Ampk activation and glucose uptake is conserved in primary human myocytes [72]. Perhaps more important the effect is preserved in vivo. Mice under constant cyclopamine infusion show robust, insulin-independent glucose uptake in skeletal muscle and BAT [72]. A single Cyclopamine bolus can equally clear blood glucose in overt, insulin-deficient, type 1 diabetic animals [72], highlighting potential for Hedgehog selective partial agonists as glucose lowering agents. In the context of cancer, marked muscle spasms and weight loss marred the first successes of BCC patient therapy with Vismodegib [138]. Recent evidence suggests that these prominent and unforeseen side-effects of Vismodegib treatment are largely prevented by combined therapy with L-Type Ca^{2+} channel inhibitors, strong indication that the side-effects are non-canonical derived (*personal communication*). These findings provide tools and a platform for reassessing and validating optimal Smo-targeting strategies (Fig. 3).

4. Conclusions and future directions

In the context of metabolic control, the next big question certainly remains physiological relevance. Our own preliminary data suggest that a fully functional endogenous ligand-receptor axis is active in vivo, one that does indeed link substrate utilization and whole body energy homeostasis (*unpublished*). The findings suggest Hedgehog indeed comprises a novel endocrine regulator of metabolic homeostasis.

The studies above suggest that much is still to be learned. In particular, we may have just struck the tip of an iceberg of regulatory potential. We would predict that while not all cells will respond with glucose uptake, every Smo expressing cell with a primary cilium likely has its own physiological output for rapid acute Smo-coupled non-canonical signalling. If true, there are some exciting discoveries to come.

Abbreviations

GLI1/2/3	glioma-associated oncogene homologue 1/2/3
PTCH	patched
SMO	smoothened
SUFU	suppressor of fused
CDO	CAM-related/downregulated by oncogenes
BOC	brother of CDO
GAS1	growth arrest-specific 1
PKA	protein kinase A
GSK3β	glycogen synthase kinase 3 β
CK1	casein kinase 1
SAG	smoothened agonist
SHH	sonic Hedgehog
DHH	desert Hedgehog
IHH	Indian Hedgehog
GRK2	G-protein coupled receptor kinase 2
PTX	pertussis toxin
RAC1	Ras-relatedC3 botulinum toxin substrate 1
RHOA	Ras-homolog family member A
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
CARMA1	CARD-containing MAGUK protein 1
BCL10	B-cell CLL/lymphoma 10
MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1

LRP2	LDL-receptor-related-protein 2
VLDL	very low-density lipoprotein
HIF1α	hypoxia-inducible factor 1 α
IGF1	insulin-like growth factor 1
IPF1/PDX1	insulin promoter factor 1/pancreatic and duodenal homeobox 1
PPARγ	peroxisome proliferator-activated receptor gamma
CAMKK2	calcium-calmodulin-dependent kinase 2
AMPK	AMP-dependent kinase
PKM2	pyruvate kinase M2 isoform
PDHa1	pyruvate dehydrogenase a1
CLD-Smo	ciliary localization deficient Smo
LKB1	liver kinase B1

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Fig. 1.

Fig. 2.

Fig. 3.